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# Glycine Betaine synthesis and transport Proteins, BetTIBA and ProPU, in *Escherichia coli* K12 do not confer Resistance to SDS-EDTA induced Outer Membrane Stress

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**SUMMARY** Glycine betaine is a small organic molecule derived from choline that acts as an osmoprotectant in *Escherichia coli* responsible for providing cellular stability during environmental stress. It is synthesized by biosynthetic proteins encoded in the *betTIBA* operon and transported in and out of the cell by the ProP and ProU transporters. Among its various functions, it is also known to protect the cell against abiotic stress factors by acting as a chemical chaperone to prevent protein denaturation. Knowing that glycine betaine can perform a myriad of functions, we were interested in determining whether this osmolyte confers resistance to outer membrane stress in *E. coli*. Here, we attempted to determine if *E. coli* that is unable to synthesize glycine betaine, due to a *betTIBA* deletion and/or *proPU* deletion, would affect cell susceptibility to SDS-EDTA induced outer membrane stress by assessing cell viability. Cell viability was measured by determining growth of the wild-type, Δ*betTIBA*, and Δ*betTIBA*Δ*proPU* mutants over a 16-hour period through $OD_{600}$ readings using the EpochTM Microplate Spectrophotometer (Biotek). Results from growth assays under SDS-EDTA conditions showed that *betTIBA* and *proPU* deletions resulted in decreased susceptibility to SDS-EDTA relative to the no SDS control. The results suggest that glycine betaine does not contribute to protecting the outer membrane from stress factors such as SDS and EDTA.

## INTRODUCTION

Glycine betaine is a water soluble, modified amino acid that contains three methyl groups and functions as an osmolyte (1, 2). It can be classified as an alkylamine, an amino acid derivative, that is produced at extremely high concentrations without perturbing cellular functions due to its hydrophilic properties and dipolar characteristics (1, 2). This osmolyte is one of the most active, naturally occurring osmolytes that prevents water efflux when subjected to hyperosmotic pressure. It has also been shown to be responsible for preventing protein aggregation (3), desiccation resistance (4), and many other osmoregulatory functions. During states of stress, glycine betaine in the cell surrounds the proteins along with the hydration shell as a protective layer. This allows for hydrophobic interactions between the protein and the osmolyte to occur to prevent denaturation of the protein (5). Osmoprotection is a ubiquitous strategy used by many mammals, plants, fungi, and microbes (6). However, glycine betaine is predominantly used by bacteria to allow them to grow in a hypertonic salt environment (2).

Glycine betaine can be synthesized from choline in a two-step process. Microbes acquire choline from the environment which is oxidized to an intermediate compound known as betaine aldehyde by choline dehydrogenase (BetA). Betaine aldehyde dehydrogenase, an essential oxidoreductase enzyme encoded by the gene *betB*, reduces betaine aldehyde to glycine betaine (5). The *bet* operon also encodes for many other proteins that assist the synthesis of glycine betaine: *betT* encodes a proton-regulated choline transporter that carry choline into the cell, *betC* encodes a choline sulfatase which catalyzes the conversion of choline-*O*-sulfate or phosphorylcholine into choline, and *betI* encodes for a transcriptional repressor that regulates *bet* operon gene expression in the presence of choline (7).

Not only can glycine betaine be synthesized, but it can also be taken-up from the environment. For Gram-negative enteric bacteria like *Escherichia coli* and *Salmonella*

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*typhimurium*, they have two systems, ProP and ProU, that can uptake glycine betaine. ProP is a part of the major facilitator superfamily (MFS) of permeases that functions as a proton symporter, while ProU is part of the ATP-binding cassette (ABC) protein superfamily that functions as an ATP dependent protein transporter (8). The *proU* operon is composed of three genes: *proV*, *proW*, and *proX*. The *proV* gene encodes for an energy-coupling component that hydrolyzes ATP and forms a complex with the inner membrane protein ProW. The *proX* gene encodes a periplasmic protein that binds to osmoprotectants and delivers it to the ProVW complex for transport across the cytoplasmic membrane (9). Both ProP and ProU have broad substrate specificity and can also take up a variety of osmoprotectants, such as proline betaine, choline, taurine, and ectoine (10).

A study on *E. coli* lacking the *otsA* gene exposed to SDS-EDTA was conducted by Chang *et al.* (11) to elucidate whether this mutant *E. coli* has increased susceptibility to abiotic stress (i.e. SDS). The *otsA* gene encodes for an enzyme involved in the trehalose synthesis pathway which is upregulated in response to osmotic stress in *E. coli*. Similar to glycine betaine, trehalose is another osmoprotectant that can protect the integrity of proteins and cellular membranes during various stress conditions. They concluded that mutants with *otsA* deletion had increased susceptibility to SDS-EDTA, suggesting that the osmolyte may play a role in outer membrane stability. However, it was also noted that glycine betaine may have been responsible for compensating the loss of trehalose, which resulted in an unexpected increased resistance in certain stress conditions. Hence, the genes encoding glycine betaine synthesis enzymes should be removed to accurately predict the effects of *ostA* deletion. Currently, there is a lack of understanding behind the mechanism of action of how glycine betaine offers protection to the cell under stressed condition. Knowing that osmolytes can perform a myriad of functions, we were interested in determining whether glycine betaine conferred resistance to outer membrane stress induced by SDS-EDTA. We are hoping that this preliminary research project can lay a foundation in determining the mechanism of action of glycine betaine for future research.

Here, we seek to determine whether the proteins involved in glycine betaine synthesis and transport proteins (i.e. BetTIBA and ProPU*)* can contribute to withstanding stress induced by SDS-EDTA. Sodium dodecyl sulfate (SDS) is an anionic detergent that can disrupt biological membranes (12), and ethylenediaminetetraacetic acid (EDTA) is a chelating agent that can sequester calcium ions associated with LPS (13). Together they can destabilize the outer membrane of *E. coli* resulting in cell lysis. Three *E. coli* strains: wild-type (MG1655), Δ*betTIBA* (MC4100), Δ*betTIBA*Δ*proPU* (MKH13) were subjected to SDS and EDTA treatment, and growth was measured. This assay was previously developed by Hartstein *et al.* (14) as a means to assess outer membrane stability in *E. coli*. Given the proposed roles of glycine betaine as an osmoprotectant in *E. coli,* we hypothesize that the *ΔbetTIBA* and Δ*betTIBA*Δ*proPU* strains, lacking proteins associated with glycine betaine synthesis and transport, would be more susceptible to SDS-EDTA treatment compared to wild-type *E. coli*.

## METHODS AND MATERIALS

***E. coli* strains**. Three *E. coli* K12 strains (described in Table 1) were used throughout the course of this project. All three strains contain no antibiotic cassettes and were grown in LB media.

**Genotypic confirmation by PCR and gel electrophoresis**. Due to uncertainty of the gene deletion, two sets of primers were designed to flank either the *betTIBA* or *betBA* operon (described in Table 2) - the same reverse primer was used for both sets.

Genomic isolation and purification were performed on MG1655, MC4100 and MKH13 with PureLink™ Genomic DNA Mini Kit (Invitrogen) and PureLink™ PCR Purification Kit (Invitrogen) respectively. The concentration and purity of isolated genomic DNA were determined using a NanoDrop3000 spectrophotometer. Isolated DNA was subjected to PCR amplification according to instructions given by Platinum™ Taq DNA Polymerase (Invitrogen) using T100 thermocycler (Bio-Rad Laboratories). PCR amplification was optimized with an initial denaturation step (2 minutes at 95°C), followed by 30 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 55°C), and extension (90

**TABLE. 1 *E. coli* strains used in this study.** Strains were obtained from Dr. Erhard Bremer's lab in Philipps University of Marburg.

| Strain ID | Genotype | Source |
|---|---|---|
| MG1655 | Wild-type, parent strain of MC4100 and MKH13 | (26) |
| MC4100 | Δ*betTIBA* | (27) |
| MKH13 | Δ*betTIBA*Δ*proPU* | (18) |

seconds at 72°C). A 1.5 % agarose gel stained with 1x SYBR® Safe DNA (Invitrogen) gel stain was prepared to resolve the PCR amplicon, and the gel was imaged with UV light on the ChemiDoc™ MP Imaging System (Bio-rad Laboratories).

**Cell Viability Assay under SDS-EDTA Conditions**. Overnight cultures of MG1655, MC4100 and MKH13 were diluted 1:200 and incubated at 37°C for 3 hours to reach mid-logarithmic growth phase. Each strain was inoculated in triplicate in a 96-well plate in LB supplemented with EDTA at a final concentration of 0.1mM. Increasing concentrations of SDS were added to each triplicate to achieve concentrations of 0.01%, 0.02%, 0.03%, 0.04%, 0.06%, 0.08%, and 0.1%. LB media without SDS was used as a negative control. The plates were grown overnight for 16 hours at 37°C. Growth was measured using OD600 readings obtained using the EpochTM Microplate Spectrophotometer (Biotek) every hour for the 16 hour period in order to generate a growth curve for the different strains.

## RESULTS

**Verification of *betTIBA* gene deletion in MC4100 and MKH13 strains**. The band sizes were expected to be either 500bp for a *betTIBA* deletion or 300bp for a *betBA* deletion. Agarose gel electrophoresis of the PCR amplicons from both strains resulted in 500bp bands, which confirmed the deletion for both strains to be the full *betTIBA* operon. No DNA template was added to the negative control. However, in order to confidently attribute any observations we made in our experiments to the gene deletions in question, it was necessary to confirm that the mutant strains have the correct deletions with Sanger sequencing. Unfortunately, due to time constraints, we were unable to obtain a conclusive sequencing result on whether the correct gene deletions were present, and thus had to operate on the assumption that the genes were correctly deleted. Future studies on MC4100 (Δ*betTIBA*) and MKH13 (Δ*betTIBA*Δ*proPU*) should ideally confirm the deletion of *betTIBA* and *proPU* before moving on to downstream experiments. This can be done by amplifying the deleted region using PCR, with primers that bind downstream and upstream of the operon. The PCR product can then be used for agarose gel electrophoresis to see if the product has the correct length in base pairs, purified, and used for sequencing.

**TABLE. 2 Primers used in this study for PCR verification of betTIBA or betBA genes deletion. Primer sequences and their corresponding directionality and melting temperatures are shown.**

| Primer Location | Primer sequence (5' - 3') | Melting Temperature (℃) |
|---|---|---|
| Upstream of *betTIBA* operon | Forward: TGCCTGTCCCTTAAAAACCCA | 59.8 |
| Upstream of *betB* gene (within *betI*) | Forward: CTGTCGAATCTGGTGAGCGA | 59.8 |
| Downstream of *betTIBA* operon | Reverse: GCCCGCATCATGAATACTGG | 59.1 |

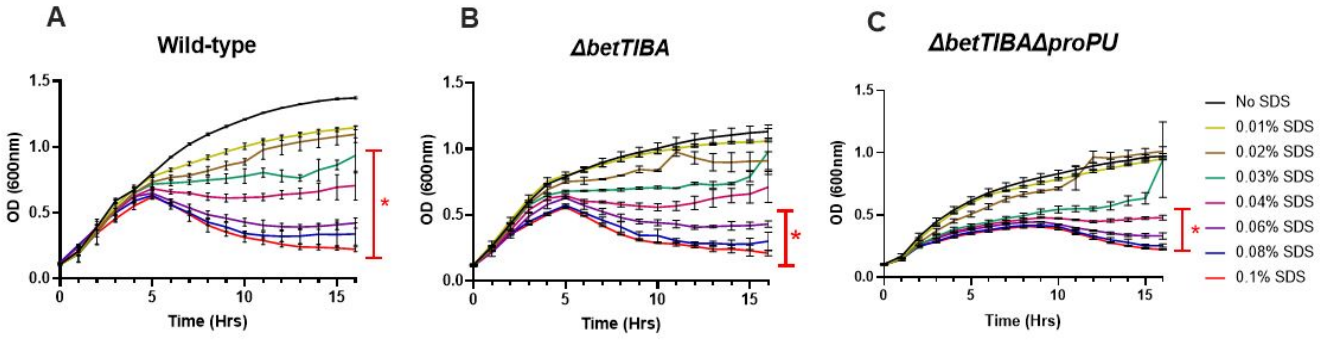
**Both mutants, MC4100 and MKH13, were less susceptible to SDS than wild-type** . To investigate the effect of *betTIBA* and *proPU* deletion in *E. coli* when presented with outer membrane stress induced by SDS-EDTA, growth curves were generated for wild-type (MG1655), Δ*betTIBA* (MC4100), and Δ*betTIBA*Δ*proPU* (MKH13) cells. EDTA concentration was kept constant at 0.1mM with increasing SDS concentrations from 0% to 0.1%. Based on the no SDS control growth curves, Δ*betTIBA* and Δ*betTIBA*Δ*proPU* have inherently lower growth compared to the parent wild-type strain. We also see the expected decreased in growth as SDS is increased, consistent in all the strains. Figure 1 shows that the WT started to have significant differences ($p<0.0001$) in growth starting at 0.03% SDS, whereas Δ*betTIBA* began to exhibit significant differences in growth when treated with 0.06% SDS, and Δ*betTIBA*Δ*proPU* at 0.04%. Thus, a higher concentration of SDS was required to affect the growth of both mutants compared to the wild-type. Therefore, contrary to expected results, the deletion of *betTIBA* resulted in increased tolerance to SDS-induced stress compared to a double-deletion of *betTIBA* and *proPU*, both of which showed increased tolerance compared to the wild-type.

## DISCUSSION

Figure 1 shows that Δ*betTIBA* and Δ*betTIBA*Δ*proPU* had lower inherent growth compared to the wild-type parent strain when grown in LB media. This suggests that the loss of glycine betaine biosynthesis and transport proteins was deleterious to growth under regular conditions in LB media. Fructose-1,6-bisphosphate aldolase (FBA) is an enzyme present in *E. coli* and is essential to glycolysis and gluconeogenesis. A recent study showed that glycine betaine moderated the inhibitory effects of salt stress on FBA in halotolerant cyanobacteria (15). Furthermore, glycine betaine can stabilize proteins to prevent aggregation both in vitro and in *E. coli* (16-17). Given the evidence that glycine betaine is capable of interacting with crucial cellular processes, it is possible that optimal growth cannot be achieved without the ability to accumulate glycine betaine. Moreover, the ProPU transporters can uptake multiple osmoprotectants besides glycine betaine such as proline, ectoine, and taurine (18) which may also be important for cell growth. Another possible explanation for lower inherent growth is that the mutants lack a major osmoprotectant system which may make them more susceptible to the osmotic stress induced by the LB media (19).

Figure 1 also shows a sudden spike in growth in all three strains grown at 0.02% SDS which can be seen at the 11-hour time point. Supposing that this observation is due to biological reasons and not technical errors, then the strains may have engaged in delayed growth resumption (20), or tried to rescue themselves by perhaps upregulating certain genes

**FIG. 1 Concentration-dependent effect of SDS on mutants deficient in glycine betaine production and uptake.** Mid-logarithmic growth phase **A)** MG1655 (WT), **B)** MC4100 (Δ*betTIBA*), and **C)** MKH13 (Δ*betTIBA*Δ*proPU*) cells were seeded into a 96 well plate and incubated at 37°C for 16 hours. The above SDS concentrations and 0.1 mM EDTA were added to the test conditions and no SDS was added to the control conditions. Growth was measured every hour to generate the curves. Error bars represent standard error of technical replicates (n=3). Growth at the 16-hour time-point for each SDS concentration was compared to the no SDS control using one-way ANOVA followed by Dunnett's multiple comparisons test. Statistically significant difference ($p<0.0001$) in growth is indicated by a red asterisk.



or processes. Of course, there is no conclusive evidence to support the latter idea in this study alone, but future studies can look to see if this spike in growth is related to any survival or SDS resistance related genes. Delayed growth resumption occurs when new resources become available to the stationary phase cells, which allow them to resume growth and division (20).

Our original hypothesis was that a deletion in the glycine betaine related genes would increase cell susceptibility to outer membrane stress such as SDS-EDTA. However, based on our SDS-EDTA assay results (Figure 1), it appears that glycine betaine synthesis increased susceptibility in the presence of SDS-EDTA. We have proposed two potential explanations for these observations. First, the lack of glycine betaine may have caused an overexpression of other osmoprotectants. A previous paper showed that in the absence of glycine betaine, the amount of trehalose increased in response to an increase in NaCl concentrations (21). Also, when glycine betaine is allowed to accumulate in the cell, trehalose synthesis is halted (22). A previous study showed that *E. coli* with a deletion in *otsA* (involved in the synthesis of trehalose) had increased susceptibility to SDS-EDTA (11). It is possible that an overexpression of trehalose in the mutants provided increased protection to SDS-EDTA compared to the WT and the accumulation of glycine betaine in the WT prevented the synthesis of trehalose, resulting in increased susceptibility to SDS-EDTA. Second, SDS resistance is energy dependent (23). If the *betTIBA* operon and *proPU* do not play a role in protection against SDS, then it is possible that an increase in SDS resistance in the mutants is due to a decrease in energy expenditure. Increased gene expression of the *betTIBA* operon, *proP*, and *proU* incur energy costs, and so do the synthesis and transport processes these genes regulate (24). This is especially true for the ATP-dependent ProU transporter.

**Limitations** In our study, we conducted our experiment using three technical replicates. Technical replicates are used to represent variations in our protocols while biological replicates are used to represent the random variations in the biological samples. Having both types of replicates would have been ideal to isolate for variations in our experiment and increase confidence in our data. However, due to time constraints, only one biological replicate was run with three technical replicates. Lastly, throughout the course of the project, we assumed that choline, the precursor molecule required for the synthesis of glycine betaine, was present in the growth media in small amounts. However, it is possible that our cells were not able to synthesize enough glycine betaine due to insufficient or lack of choline. A minor modification to our current protocol would be the addition of choline to the media.

**Conclusions** We examined the role of glycine betaine biosynthesis genes, *betTIBA* and glycine betaine transporter genes *proPU* on outer membrane stress through an SDS-EDTA susceptibility assay. Knowing that osmolytes can perform a myriad of functions and the effects of glycine betaine on outer membrane stability under abiotic stress are limited, we were interested to determine whether glycine betaine confers resistance to outer membrane stress induced by SDS-EDTA. We originally hypothesized that *ΔbetTIBA* and Δ*betTIBA*Δ*proPU* strains would be more susceptible to SDS-EDTA treatment compared to wild-type *E. coli*. However, data showed inconclusive results as mutants deficient in both glycine betaine production and transport seemed to be affected by SDS-induced stress faster but ultimately showed increased tolerance to higher concentrations of SDS compared to the wild-type.

**Future Directions** Due to the compensatory nature of trehalose and glycine betaine (21), an *E. coli* strain lacking both glycine betaine and trehalose synthesis enzymes along with its corresponding transporters should be examined under SDS-EDTA induced outer-membrane stress in the future to be able to confirm whether a deficiency in these osmoprotectants will increase susceptibility to membrane disruption reagents.

In another study, researchers discovered that the primary accumulating osmolyte changes depending on the salt concentrations (25). Relating this to our study, it is possible for SDS to trigger the accumulation of another osmolyte beside glycine betaine. It is also

probable that more than one osmolyte was used in response to SDS-EDTA induced membrane disruption. Thus, performing qPCR on other stringent response genes, such as *otsA*, *spoT*, and *relA* would be a reasonable experiment moving forward. This can demonstrate whether other genes were upregulated to compensate for the loss of glycine betaine, which could explain the surprisingly high resistance of mutant strain to SDS in our results.

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